Characterization of the *Xanthomonas campestris* pv. *campestris* Lipopolysaccharide Substructures Essential for Elicitation of an Oxidative Burst in Tobacco Cells

Sebastian G. Braun,¹ Andreas Meyer,¹ Otto Holst,² Alfred Pühler,¹ and Karsten Niehaus¹

¹Fakultät für Biologie, Lehrstuhl für Genetik, Universität Bielefeld, Postfach 100131, D-33501 Bielefeld, Germany; ²Research Center Borstel, Analytical Biochemistry, D-23845 Borstel, Germany

Submitted 4 October 2004. Accepted 4 March 2005.

The lipopolysaccharides (LPS) of gram-negative bacteria are essential for perception of pathogens by animals and plants. To identify the LPS substructure or substructures recognized by plants, we isolated water-phase (w)LPS from different *Xanthomonas campestris* pv. *campestris* mutants and analyzed their sugar content and ability to elicit an oxidative burst in tobacco cell cultures. The different wLPS species are characterized by lacking repetitive subunits of the O-antigen, the complete O-antigen, or even most of the core region. Because loss of lipid A would be lethal to bacteria, pure lipid A was obtained from *X. campestris* pv. *campestris* wild-type wLPS by chemical hydrolysis. The elicitation experiments with tobacco cell cultures revealed that LPS detection is dependent on the bioavailability of the amphiphilic wLPS, which can form micelles in an aqueous environment. By adding deoxycholate to prevent micelle formation, all of the tested wLPS species showed elicitation capability, whereas the lipid A alone was not able to trigger an oxidative burst or calcium transients in tobacco cell cultures. These results suggest that the LPS substructure recognized by tobacco cells is localized in the inner core region of the LPS, consisting of glucose, galacturonic acid, and 3-deoxy-d-manno-oct-2-ulosonic acids. Although lipid A alone seems to be insufficient to induce an oxidative burst in tobacco cell cultures, it cannot be ruled out that lipid A or the glucosamine backbone may be important in combination with the inner core structures.

Additional keywords: nonhost resistance, pathogen-associated molecular patterns (PAMPs).

Xanthomonas campestris pv. *campestris* is a gram-negative, phytopathogenic bacterium causing the black rot disease of brassicaceen. The symptoms of this disease on susceptible plants are black-colored veins and rotting leaves. Nonhost plants resistant to *X. campestris* pv. *campestris* infection (e.g., tobacco or bell pepper) respond to *X. campestris* pv. *campestris* by producing reactive oxygen species (ROS), e.g., H₂O₂ at the infection site, the induction of pathogen-related (PR) gene expression, and biosynthesis of secondary plant metabolites, namely phytoalexins involved in the resistance response (Hammerschmidt 1999). This leads to the death of the plant

cells and of the infecting bacterial cells. In case of a rapid cell death, this reaction is also termed the hypersensitive response (HR). All plant species not infected by *X. campestris* pv. *campestris* are called nonhost plants of *X. campestris* pv. *campestris* because of their ability to recognize the bacteria and, in consequence, defeat the infection. Recognition is mediated by different chemical compounds that derive from the invading microbe or are released from the degraded plant cell walls (Heath 2000; Nürnberger et al. 2002; Radman et al. 2003). These substances are commonly termed elicitors, due to their ability to elicit plant defense reactions. Some of the known elicitors in the *Xanthomonas*-plant interaction are secreted proteins (flagellin, Felix et al. 1999), avirulence proteins (AvrBr3, Van den Ackerveken et al. 1997), degradation products of the plant cell wall (oligogalacturonides, Scheidle et al. 2005), and lipopolysaccharides (Dow et al. 2000; Meyer et al. 2001; Newman et al. 2002).

Lipopolysaccharides (LPS) are unique to gram-negative bacteria and are located in the outer membrane. Typically, the LPS molecule consists of three major substructures, the O-antigen or O-chain, the core region, and the membrane-anchored lipid A (Raetz et al. 2002). The O-antigen is composed of a repetitive sugar subunit. Characteristically, the degree of polymerization of this repeating unit is between 20 and 50. The O-antigen is connected to the core region consisting of a sugar chain, which is linked via one or more 3-deoxy-D-manno-oct-2-ulosonic acids (Kdo) or a D-manno-oct-2-ulosonic acid with the last part of the molecule, the lipid A. In the typical composition, the lipid A consists of a glucosamine-phosphate backbone with ester- and amide-bound fatty acids (Holst and Brade 1992).

Due to the ability of the nonhost or, in case of human pathogenic bacteria, of the host to detect gram-negative bacteria on the basis of their LPS, bacteria developed a wide spectrum of compositionally different LPS (Ojanen et al. 1993). In case of the human-pathogenic bacteria, the O-chain functions as an important antigen and is used in serotyping of gram-negative pathogens. The core and the lipid A region, which is the part containing the endotoxin activity in human pathogens (Raetz et al. 2002), of different species and pathovars can vary in structure (Holst 2002; Molinaro et al. 2003; Takayama et al. 1986). Variations in lipid A consist mainly of different fatty acid compositions and lead to a heterogeneity even in the same bacterium (Qureshi et al. 1985).

Experiments with *X. campestris* pv. *campestris* and its non-host plant *Capsicum annuum*, in which the plant leaf was pre-

Corresponding author: K. Niehaus; E-mail: Karsten.Niehaus@Genetik.Uni-Bielefeld.de; Telephone: +49 (0)521 106-5631; Fax: +49 (0)521 106-5626.

inoculated with *X. campestris* pv. *campestris* LPS and LPS derivatives, showed a reduced defense reaction (Newman et al. 1997). This result indicates that the LPS region responsible for this effect is located somewhere in the core.

To identify the substructures of the LPS molecule responsible for the biological functions, we analyzed different *X. campestris* pv. *campestris* LPS types obtained by mutagenesis of LPS biosynthesis genes or chemical cleavage of wild-type LPS. The LPS species were further characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and sugar compositional analysis. Tests for elicitor activity were performed in an oxidative burst assay using tobacco cell cultures and showed that the activity is correlated with the glucose-galacturonic acid oligosaccharide.

RESULTS

Defined *X. campestris* pv. *campestris* mutants yield different LPS types.

Defined *X. campestris* pv. *campestris* mutants defective in the LPS biosynthesis and chemical cleavage of wild-type LPS yield different LPS types, varying in length of the oligosaccharide chain. In order to define the parts of the *X. campestris* pv. *campestris* LPS molecule that are recognized by the nonhost plant tobacco, truncated LPS variants were required. Since there is no known procedure to obtain defined sequential LPS substructures by chemical methods, mutants of *X. campestris* pv. *campestris* that synthesize truncated forms of LPS were used. Due to the fact that mutants resulting in LPS composed only of lipid A seem not to be viable, lipid A was obtained chemically. For this purpose, *X. campestris* pv. *campestris* wild-type LPS was hydrolyzed at 100°C, using acetic acid to cleave off the core oligosaccharide at the Kdo linker. Due to its hydrophobic nature, the lipid A is easily separated from the oligosaccharides and is further purified by ultracentrifugation.

Water-phase (w)LPS of the *X. campestris* pv. *campestris* wild-type strain B100, the mutants H14, H2321, and H23.8 (Vorhölter et al. 2001), as well as the lipid A were analyzed by SDS-PAGE and silver staining. Major changes in the LPS structure are easily detectable by this method. The wild-type wLPS of *X. campestris* pv. *campestris* showed one diffuse slow-migrating and two well-defined faster-migrating bands, when analyzed by SDS-PAGE (Fig. 1A). The slow-migrating band corresponds to the entire wLPS, composed of the lipid A core–O-chain moiety. The upper band of the faster-migrating bands represents the lipid A–core and the lower band the lipid A–inner core parts of the wLPS (Vorhölter et al. 2001). The mutant H2321 lacked the diffuse slow-migrating band, corresponding to the entire wLPS molecule containing a polymeric O-antigen. After increasing the sample concentration in SDS-PAGE, an additional band appeared (Fig. 1B). Obviously, the H2321 mutant has no complete O-chain but has a shortened or altered one. Most probably, the wLPS of H2321 carries only one repeating unit of the O-antigen, while mutant H23.8 showed no O-chain at all (Fig. 1C). In this case, only the lipid A–core wLPS is formed. The mutant

H14 (Fig. 1D) lacked the O-chain and the outer core part of the wLPS in the SDS-PAGE. There was only one band left that migrated even faster than the lower band of the wild-type double bands, which indicates that the inner core is incomplete and lacks one or more sugar molecules. A comparison of the band of the H14 mutant with that of the lipid A (Fig. 1E) showed that there was no detectable difference in migration distance in the SDS-PAGE. Obviously, the H14 mutant wLPS lacks almost the entire core part.

Sequential reduction in heterogenicity resulted in four different LPS species.

The monosaccharide composition of *X. campestris* pv. *campestris* wild-type and mutant strains wLPS showed a sequential reduction in heterogenicity resulting in four different LPS species. The different wLPS of the wild-type *X. campestris* pv. *campestris* B100 and mutants H2321, H23.8, and H14 were analyzed for their monosaccharide composition. The wild-type wLPS consists of rhamnose, mannose, glucose, glucosamine, and galacturonic acid (Table 1). The wLPS of the H2321 mutant showed the same monosaccharide composition but a reduced amount of rhamnose. Based on the observation in the SDS-PAGE, the lower amount might be due to a reduced O-chain part of the wLPS. The H23.8 mutant, lacking the entire O-chain, showed no detectable rhamnose in the gas chromatography-mass spectrometry (GC-MS) analysis (Table 1).

Fig. 1. Comparison of the water-phase lipopolysaccharide (wLPS) pattern of wild-type and different mutant LPS species in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis. **A**, *Xanthomonas campestris* pv. *campestris* B100 wLPS shows the typical three bands, one corresponding to a high molecular weight LPS (O-antigen, core, and lipid A), two migrating close together and corresponding to low molecular weight LPS (probably comprising lipid A plus whole core and inner core, respectively). **B**, Mutant H2321 shows the low molecular weight double band but not the high molecular weight band. By overloading the gel, new bands become visible, migrating between the missing high molecular weight and the two low molecular weight bands. **C**, Mutant H23.8 only shows the fast-migrating double band. **D**, The wLPS mutant H14 lacks the usual bands but shows a new band migrating even farther than the bands of the wLPS species. **E**, The chemically obtained lipid A shows one band migrating approximately as far as the H14 wLPS.

Table 1. Sugar analysis of four different *Xanthomonas campestris* pv. *campestris* lipopolysaccharides (LPS) species^a

Monosaccharides	<i>X. campestris</i> pv. <i>campestris</i> LPS species			
	B100	H2321	H23.8	H14
Rhamnose	210 (1.1)	173 (0.5)	–	–
Mannose	256 (1.3)	516 (1.4)	213 (1.6)	–
Glucose	204 (1.0)	340 (1.0)	143 (1.1)	114 (1.0)
Galacturonic acid	200 (1.0)	364 (1.0)	133 (1.0)	120 (1.0)

^a Monosaccharides were prepared by methanolysis and were analyzed as aldetol acetates by gas chromatography-mass spectrometry. The absolute values are given as nmol sugar per mg of the corresponding water-phase (w)LPS. Because of the different molecular weights of the wLPS species, relative values were determined (in brackets). Based on the absolute value of galacturonic acid, fractions or multiples of the other sugars were determined.

Otherwise, the monosaccharide composition was comparable to that of the wild-type B100 and H2321 wLPS. The band shift of the H2321 wLPS in SDS-PAGE and the reduced amount of rhamnose, or the total loss in case of the H23.8 wLPS, showed that rhamnose is part of the O-chain of the wLPS of *X. campestris* pv. *campestris*. In the wLPS obtained from mutant H14, there was no mannose present, and only reduced amounts of glucose and galacturonic acid could be detected (Table 1).

The compositional analysis combined with SDS-PAGE lead to the models shown in Figure 2. The wLPS of *X. campestris* pv. *campestris* B100 consists of a rhamnan O-chain and the core oligosaccharide of mannose, glucose, and galacturonic acid, whereas H2321 has the same composition but with less rhamnose, suggesting a shortened O-chain. Mutant H23.8 seems to lack the O-chain completely but to have the other sugars in comparable amounts to the wild-type B100 wLPS. The wLPS of H14 not only lacks the O-chain but also mannose, resulting in an inner core consisting of only glucose and galacturonic acid, linked via Kdo to the lipid A.

Bioavailability correlates with reduced elicitor activity of wLPS mutant H2321 in an aqueous solution.

In tobacco cell cultures, the reduced elicitor activity of wLPS mutant H2321 in an aqueous solution correlates with its bioavailability. *X. campestris* pv. *campestris* wLPS is able to trigger an oxidative burst in tobacco cell cultures, as shown by Meyer and associates (2001). Yeast invertase was used as a positive control in the experiments, since it induced a strong oxidative burst reaction as compared with that induced by the less-defined yeast elicitor (Baier et al. 1999; Meyer et al. 2001) but with the advantage of not being a mixture of substances (Trimble et al. 1983).

As shown previously by Meyer and associates (2001), addition of 20 µg of wLPS isolated from the wild type B100 per milliliter to the tobacco cells resulted in the induction of an oxidative burst reaction (Fig. 3). The intensity of the reaction

was comparable to that induced by invertase, even though a characteristic delay of approximately 10 min in the onset was observed (Fig. 3). In contrast, wLPS of the mutant H2321 induced an even more delayed and weaker oxidative burst in the tobacco cell cultures. The remaining activity was surprising, since structural modifications on a recognized chemical epitope will generally result in a drastic loss of activity, e.g., as demonstrated for the *Phytophthora megasperma* oligosaccharide elicitor (Ebel et al. 1976). For this reason, it appeared more likely that the shortened O-chain of the H2321 wLPS contributed to a reduced bioavailability of the elicitor.

To test if the amphiphilic nature of wLPS correlates with the quantity of the elicitor activity, a batch of the wild-type and mutant wLPS, respectively, was dissolved in a deoxycholate (DOC) solution. DOC acts as a detergent and prevents the aggregation of the wLPS and, therewith, increases its bioavailability. With this treatment, the oxidative burst of the wild-type B100 wLPS could be improved (Fig. 4). Comparing the oxidative burst reaction induced by the mutant H2321 and the wild-type B100, elicitor activity of the truncated H2321 wLPS could be nearly restored by the addition of DOC (Figs. 3 and 4). This indicated that reduced elicitor activity of H2321 wLPS was not an effect of a lost recognition site but of reduced bioavailability due to nonoptimal solubility.

Elicitor activity is correlated with the glucose-galacturonic acid-Kdo oligosaccharide found in the inner core.

The elicitor activity of the *X. campestris* pv. *campestris* wLPS is correlated with the glucose-galacturonic acid-Kdo oligosaccharide found in the inner core. To determine the wLPS domain required for the recognition of the *X. campestris* pv. *campestris* wLPS, further wLPS derivatives were tested in tobacco cell culture for their elicitor activity. To rule out the possibility that the loss of the elicitor activity was caused by a reduced bioavailability, all wLPS samples were dissolved in a

Fig. 2. Lipopolysaccharide (LPS)-structure models based on the sugar composition analysis. LPS-structure models of *Xanthomonas campestris* pv. *campestris* B100 wild-type water-phase (w)LPS and different *X. campestris* pv. *campestris* mutant wLPS derived from gas chromatography-mass spectrometry monosaccharide analyses. The *X. campestris* pv. *campestris* B100 wLPS model shows the typical LPS structure composed of the O-chain, consisting of rhamnose, a core region consisting of mannose, glucose, and galacturonic acid, and a lipid A linked to the core via Kdo. The “n” indicates the unknown number of O-antigen repeats. The H2321 mutant wLPS has a reduced amount of rhamnose, indicating fewer repeats in the O-antigen, represented by the “?”. Apart from this, the structure appears to be the same as the wild-type wLPS of strain B100. Mutant H23.8 exhibits a complete loss of rhamnose, and mutant H14 is further more truncated in the core region, missing the mannose moiety.

solution of DOC. The *X. campestris* pv. *campestris* B100 wLPS showed a 10-min delay in the onset of the oxidative burst as compared with invertase. Its maximum was reached at 30 min and dropped to zero after 60 min. In comparison with the wild-type wLPS, mutants H2321 and H23.8, lacking sugars in the O-chain part, exhibited a comparable elicitor activity (Figs. 3 and 4). Both showed the same oxidative burst kinetics, with a slightly reduced maximum in the case of mutant H23.8. After concluding that the O-chain of the wLPS seems not to be important for recognition in tobacco, the H14 mutant LPS was tested. The deep-rough mutant H14 showed a significant structural difference as compared with H2321 and H23.8 in that the O-chain was missing and most sugar compounds of the core part were lacking. It was far less able to trigger an oxidative burst than were the other two mutants. Its maximum of about 25 $\mu\text{mol H}_2\text{O}_2$ at 35 min was lower, while the general burst kinetics stayed the same. wLPS of the mutant H14 was still recognized as an elicitor by the tobacco plant cell cultures. Mutants synthesizing an even more reduced form of wLPS are not available. Most probably because a further truncated wLPS will be lethal for *Xanthomonas* cells. For this reason, wild-type B100 wLPS was hydrolyzed, resulting in lipid A. The lipid A was dissolved in DOC solution and was tested for the induction of an oxidative burst in tobacco cell suspension cultures. No oxidative burst could be detected, indicating that the pure lipid A is biologically inactive.

The onset of the oxidative burst reaction is the consequence of a complex signal transduction cascade. One of the fastest signal transduction events essential to the onset of defense reactions like the oxidative burst is a transient increase in cytosolic calcium (Mithöfer et al. 1999). To analyze the effect of the different LPS structures on the release of this second messenger, we used transgenic tobacco cell cultures expressing the bioluminescent calcium sensor aequorin. As a positive control, tobacco cell cultures were elicited with *N*-acetyl chitopentase

at a final concentration of 5 $\mu\text{g/ml}$. The bioluminescence, representing the cytosolic calcium level, was recorded for the next 10 min (Fig. 5). Approximately 1 min after the addition of the elicitor, a sharp increase in bioluminescence was observed, reaching a maximum within the next 45 s. Thereafter, luminescence decreased, passing a characteristic second but lower maximum at 3 min. In general, luminescence reached the resting level 5 min after addition of the chito-oligosaccharide.

In contrast to *N*-acetyl chitopentase, a concentration of B100 wLPS (20 $\mu\text{g/ml}$) caused a less-pronounced but significant release of calcium in the plant cytoplasm (Fig. 5). The onset of the reaction could be observed 3 to 4 min after the addition of the B100 wLPS. A first maximum was reached after 6 to 7 min, a timepoint at which the chito-oligosaccharide-induced reaction had already returned to the resting level. While the B100 wLPS induced an increase in cytoplasmic calcium levels, chemically purified lipid A (20 $\mu\text{g/ml}$) was inactive (Fig. 5). Even at a final concentration of 100 $\mu\text{g/ml}$, no significant change in bioluminescence was observed (data not shown).

These findings give strong evidence that the recognized part of the wLPS is to be found in the inner core region of the LPS molecule. The difference between the nonactive lipid A and still-active H14 wLPS is a glucose-galacturonic acid-Kdo oligosaccharide, which seems to be essential for detection as an elicitor by tobacco cell cultures.

DISCUSSION

The role of LPS as endotoxin in the animal-pathogen interaction has been extensively studied and the underlying molecular mechanisms are understood to some extent (Holst et al. 1992). The evidence accumulated during the last years showed that LPS plays a vital role in the detection of gram-negative bacteria in plant-microbe interactions also (Graham et al. 1977; Meyer et al. 2001; Newman et al. 2002). In a former

Fig. 3. An increased induction of an oxidative burst by an amphiphilic water-phase lipopolysaccharide (wLPS) mutant is achieved by the addition of deoxycholate (DOC). Tobacco cell cultures treated with either 20 μg of invertase per ml or wLPS dissolved in water were used for the elicitation reaction. Increased bioavailability of the elicitor was achieved by dissolving wLPS in a DOC solution (1mg/ml). The formation of H_2O_2 was detected using luminol-derived chemiluminescence. Yeast invertase was used as elicitation control and sole DOC solution (1mg/ml) as a negative control.

study by Meyer and associates (2001), it was demonstrated that wild-type wLPS of *X. campestris* pv. *campestris* B100 induces calcium signaling and an oxidative burst in tobacco cell cultures. As in animals, it seems possible that, in plants also, the LPS plays a general role in nonhost resistance, as one compound of the invariant pathogen-associated molecular patterns (PAMPs). If this holds true, we can speculate that, in plant-pathogenic bacteria also, a conserved substructure of the LPS is recognized by the plant (Nürnberg et al. 2004).

In this report, we made use of different *X. campestris* pv. *campestris* mutants defective in the LPS biosynthesis, to define the substructures of the LPS responsible for the induction of defense reactions on nonhost plants. Mutant H2321 carries a transposon in the gene *wxcD*. *WxcD* exhibits homologies to glycosyltransferases involved in chain length determination in LPS biosynthesis (Vorhölter et al. 2001). The proposed function of *WxcD* is in good correlation with the phenotype, the lack of O-antigen, and only traces of detectable rhamnose. From the monosaccharide compositional analysis of *X. campestris* pv. *campestris* B100 and the mutant H2321 wLPS in combination with the published structure of the O-chain of *Xanthomonas campestris* 8004 (Molinaro et al. 2003), we can assume that *X. campestris* pv. *campestris* B100 also carries a homorhamnan as O-chain. Most probably, wLPS of the mutant H2321 has only one repeat of the homorhamnan repeating unit. The mutant H23.8 carries a defective *rmlD* gene. *rmlD*, formerly known as *rfbC*, encodes a protein involved in TDP-rhamnose biosynthesis (Stevenson et al. 1994). In consequence, the wLPS of mutant H23.8 lacks all detectable rhamnose and, therefore, the entire O-chain. The mutant H14 carries a transposon in the *xanB* gene, coding for an epimerase required for the synthesis of GDP-mannose (Köplin et al. 1992). As shown here by GC-MS, no man-

nose was detectable, but glucose and galacturonic acid were detected in wLPS of this mutant. A rougher mutant has not been described so far. For this reason, pure lipid A was obtained by chemical hydrolysis from B100 wLPS.

Newman and associates (1997) performed experiments preincubating pepper leaves with *X. campestris* pv. *campestris* LPS before infecting it with live *X. campestris* pv. *campestris* bacteria. The results were infected plant leaves no longer able to initiate a HR, a so-called preventative effect (Newman et al. 1997). They suggest that the LPS binds to a limited number of receptor molecules of the plant cells, blocking them so they can no longer bind to the bacteria. Furthermore, truncated LPS species characterized by SDS-PAGE as well as core oligosaccharides were still able to prevent the HR, whereas sole lipid A or *Salmonella* LPS did not. The findings imply that the elicitor activity of the LPS is in the (inner) core region, but what compounds were actually detected remained unknown.

A truncated O-chain in wLPS resulted in a reduced induction of the oxidative burst in tobacco cell cultures. The successive removal of O-antigen and core sugar compounds inevitably led to an increase in hydrophobicity, due to the lipophilic nature of the lipid A, which promotes an increase in micelle formation (Aurell et al. 1998). To prevent micelle formation, the different LPS species were diluted in DOC and compared with corresponding samples without detergent, showing that the elicitor activity decreased with the increase of lipophilic properties of the LPS. The elicitor activity could be restored by diluting the truncated wLPS in DOC, suggesting that not the recognition site of the molecule but the ability to form micelles reduces the activity. From these experiments, we can conclude that the homorhamnan O-chain is not recognized by the plant but modifies the bioavailability of the LPS.

Fig. 4. Maximal H₂O₂ production by the different lipopolysaccharide (LPS) species in an oxidative burst assay. The diagram summarizes the oxidative burst maxima induced in tobacco cell cultures by invertase compared with the different water-phase (w)LPS species with or without deoxycholate (DOC). Mean and standard deviation of three independent replicates are given. With increased truncation of the O-antigen and core part of the wLPS, the elicitation potential, given as H₂O₂ production maximum, decreases. The effect can be nearly restored by adding DOC, showing that the elicitation decrease was due to reduced solubility and not a modified recognition site.

By comparing the elicitor activity of the different LPS species in tobacco cell cultures, the O-antigen as well as the outer core region seem to play no role in the recognition process. On the other hand, the LPS of deep-rough mutant *X. campestris* pv. *campestris* H14 showed a decreased but still measurable activity, whereas the lipid A seems to be inactive. A comparable result was obtained by determining the cytoplasmic calcium levels upon elicitation. The *N*-acetyl chitopentaose, serving as a positive reference (Mithöfer et al. 1999), induced a characteristic biphasic calcium transient. As previously reported by Meyer and associates (2001), calcium transients induced by B100 wLPS were weaker and showed no sharply pronounced peak. The isolated lipid A was not able to induce any calcium alterations. As shown in different studies (Jiang and Zhang 2003; Mithöfer et al. 1999), a calcium signal is required for the induction of the oxidative burst. Therefore, it seems evident that lipid A is not able to trigger the signal cascade involving the second messenger calcium.

The results obtained indicate that the compound vital for the recognition of the LPS lies in the inner core part of the molecule. Most probably an oligosaccharide containing glucose, galacturonic acid, and Kdo is required for the recognition by the plant.

These findings correlate well with results obtained by Newman and associates (1997) in pepper leaves, using the preventive effect. It seems logical to find the recognition site in the core region because it is not as variable as the O-antigen or the lipid A (Holst et al. 1992), giving the plant the ability to detect a broader spectrum of pathogenic *Xanthomonas campestris* strains with one receptor.

The perception of LPS has been best-studied in insects and mammals (Imler and Zheng 2003). In mammals, the innate immune response to LPS is triggered through toll-like receptor 4 (TLR4), which consists of an extracellular leucine-rich repeat domain and a cytoplasmic toll interleukin 1 receptor (toll/IL-1R/R) domain (Poltorak et al. 1998; Qureshi et al. 1999; Underhill and Ozinsky 2002). During the recognition process, LPS binds to soluble LPS-binding protein and then forms a complex with GPI-anchored protein CD14, MD-2 and the transmembrane TLR4 (Jack et al. 1997; McGuinness et al. 2003; Underhill and Ozinsky 2002; Wurfel et al. 1997). Mammals possess only about 10 TLR receptors to recognize PAMPs. In plants, more than 600 members of the family of transmembrane receptor-like kinases have been identified in *Arabidopsis thaliana* (Morris and Walker 2003; Shiu et al. 2003). Therefore, it is tempting to speculate that LPS recognition in plants is achieved by a mechanism resembling the one present in mammals. Future studies will address this hypothesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Xanthomonas campestris pv. *campestris* B100 (wild-type LPS, Sm^r [Hötte et al. 1990]), H2321 ($\Delta wxcD$, Sm^r, Km^r, Vorhölter et al. 2001), 23.8 ($\Delta rmlD$, Cm^r [Steinmann 1996]), and H14 ($\Delta xanB$, Sm^r, Km^r [Hötte et al. 1990]) were grown on tryptone-yeast (TY) agar plates (Aguilar et al. 1985) plus 0.4% (wt/vol) glucose at 30°C. The medium was supplemented with streptomycin (Sm) at a concentration of 800 μ g ml⁻¹ (*X. campestris* pv. *campestris* B100) and kanamycin (Km) at 50 μ g ml⁻¹ (*X. campestris* pv. *campestris* H2321 and H14) or chloramphenicol (Cm) at 50 μ g ml⁻¹ (*X. campestris* pv. *campestris* H23.8).

Construction of an *rmlD* mutant.

To create a deletion mutant in the *rmlD* gene (Köplin et al. 1993), formerly known as *rfbC*, the vector pK18mob (Schäfer

et al. 1994) was first digested with *KpnI*, and the overhangs were filled with Klenow polymerase and were relegated, resulting in pK18mob Δ *KpnI*. This vector was used to clone a 3.8-kb *EcoRI* fragment containing the *rml* cluster into its unique *EcoRI* site. Subsequently, an internal 107-bp *KpnI* fragment of *rmlD* was replaced by a 3.5-kb *cat* gene conferring chloramphenicol resistance. The resulting vector pDS23 was electroporated into *X. campestris* pv. *campestris* B100, and double recombinants were selected. Homogenotes were confirmed by Southern hybridization, and the mutant was termed *X. campestris* pv. *campestris* H23.8.

Isolation and purification of lipopolysaccharides.

Bacteria were grown on TY agar plates for two days. They were harvested in 3 ml of a 0.9% NaCl solution and were pooled. The pooled cell suspensions were centrifuged at 5,000 $\times$ *g* for 20 min, and the pellet was washed thrice with 200 ml 0.9% NaCl and once with 80 ml of distilled H₂O. The LPS was isolated from the resuspended cells, using the hot phenol-water method (Jann et al. 1965). The water phase was extensively dialyzed against water. Nucleic acids were removed by DNase I (100 μ g ml⁻¹) and RNase (15 μ g ml⁻¹) treatment and proteins by proteinase K (150 μ g ml⁻¹) treatment. The LPS was dialyzed again and lyophilized. The crude LPS extract was resuspended and ultracentrifuged at 100,000 $\times$ *g* for 8 h. The pellet was resuspended in H₂O and lyophilized. For biochemical and biological analyses of LPS, an aliquot was dissolved in distilled H₂O (5 mg of LPS ml⁻¹) and in a 1 mg ml⁻¹ DOC solution (2 mg of LPS ml⁻¹), respectively.

Fig. 5. Effects of different lipopolysaccharide (LPS) species on the level of cytoplasmic calcium in transgenic tobacco cell cultures carrying the apoaequorin gene. The cytoplasmic calcium was measured using calcium-dependent coelenterazine-derived chemiluminescence. Calcium signals were measured after the addition of chitopentaose, B100 wLPS, or lipid A (final concentrations of 5, 20, and 20 μ g/ml, respectively).

LPS analysis by SDS-PAGE.

The LPS species were analyzed by SDS-PAGE, using a 16.5% separating gel and a 4% stacking gel (Lesse et al. 1990). Visualizing of the LPS was done by silver staining (Kittelberger and Hilbink 1993).

Oxidative burst measurement in tobacco cell cultures.

The system used for detecting an oxidative burst was described by Warm and Laties (1982) and is based on H₂O₂-dependent chemiluminescence of luminol. *Nicotiana tabacum* cell cultures (Baier et al. 1999) were used four to five days after subcultivation, were diluted with pre-incubation medium (3% [wt/vol] sucrose in 0.04 mg/ml MS medium [Murashige and Skoog 1962] to a final concentration of 125 µg ml⁻¹), and were incubated for 3 to 4 h. To determine H₂O₂ concentrations, 200 µl of the cell suspension was mixed with 700 µl of a 50 mM K-phosphate buffer (pH 7.9), 100 µl of 1 mM luminol in K-phosphate buffer, and 100 µl of 10 mM potassium ferrocyanate. The chemiluminescence reaction was detected using a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany).

For the tests, 160 µg of LPS from a 2 mg ml⁻¹ stock solution was used for 8-ml cell suspension, resulting in a 20 µg ml⁻¹ LPS concentration in cell culture.

Detection of intracellular calcium in aequorin-transformed tobacco cell cultures.

Detection of intracellular calcium was carried out using an aequorin-transformed tobacco cell culture and methods described by Meyer and associates (2001). A 5-ml volume of the suspension cell culture was transferred to a sterile petri dish (5 cm in diameter), and 50 µl of coelenterazine (0.25 mM in ethanol) were added. Cells were incubated on a rotary shaker (125 rpm) for 6 h in the dark. For the measurements, 200 µl of the cell suspension was transferred to a cuvette and compounds were added as indicated. The chemiluminescence reaction was detected using a Sirius luminometer (Berthold Detection Systems). *N*-acetyl chitopentaose (Seikagaku, Tokyo) was used as a positive control and reference for the elicitation of the cells. *N*-acetyl chitopentaose was dissolved in distilled water. LPS (5 mg ml⁻¹) were dissolved in 50% dimethyl sulfoxide (DMSO). Prior to use, this stock solution was diluted 1:10, using distilled water to reduce the DMSO concentration. Complete discharging of aequorin was achieved by the addition of 200 µl of a solution containing 12.5% (vol/vol) DMSO and 12.5 mM CaCl₂. Discharging ensured that the concentration of reconstituted aequorin was not limiting and that the maximal consumption induced by the elicitor treatment did not exceed more than 5% of the available aequorin.

Analysis of the monosaccharide composition of the LPS by GC-MS.

To determine the monosaccharides, 500 µg of LPS of a 5 mg/ml stock solution was methanolized using 0.5 M HCl/MeOH for 45 min at 85°C. After the carboxy reduction with NaBD₄, the products and a second, parallel sample for derivatisation of only the neutral sugars were hydrolyzed with 0.1 M HCl for 48 h at 100°C. Following the carbonyl reduction and the peracetylation, the alditol acetates were analyzed by GC-MS (Trace GC, Polaris MS; Thermo Finnigan, Egelsbach).

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft SFB 549. We thank D. Steinmann for the construction of the *X. campestris* pv. *campestris* mutant H23.8 and T. Patschkowski for critically reading the manuscript.

LITERATURE CITED

- Aguilar, O. M., Kapp, D., and Pühler, A. 1985. Characterization of a *Rhizobium meliloti* fixation gene *fixF* located near the common nodulation region. *J. Bacteriol.* 164:245-254.
- Aurell, C. A., and Wistrom, A. O. 1998. Critical aggregation concentrations of Gram-negative bacterial lipopolysaccharides (LPS). *Biochem. Biophys. Res. Com.* 253:119-123.
- Baier, R., Schiene, K., Kohring, B., Flaschel, E., and Niehaus, K. 1999. Alfalfa and tobacco cells react differently to chitin oligosaccharides and *Sinorhizobium meliloti* nodulation factors. *Planta* 210:157-164.
- Dow, M., Newman, M.-A., and von Roepenack, E. 2000. The induction and modulation of plant defense responses by bacterial lipopolysaccharides. *Annu. Rev. Phytopathol.* 38:241-261.
- Ebel, J., Ayers, A. R., and Albersheim, P. 1976. Host-pathogen interaction. 12. Response of suspension-cultured soybean cells to elicitor isolated from *Phytophthora megasperma* var *sojae*, a fungal pathogen of soybeans. *Plant Physiol.* 57:775-779.
- Felix, G., Duran, J. D., Volko, S., and Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* 18:265-276.
- Graham, T. L., Sequeira, L., and Huang, T.-S. R. 1977. Bacterial lipopolysaccharides as inducers of disease resistance in tobacco. *Appl. Environ. Microbiol.* 34:424-432.
- Hammerschmidt, R. 1999. Phytoalexins: What have we learned after 60 years? *Annu. Rev. Phytopathol.* 37:285-306.
- Heath, M. C. 2000. Nonhost resistance and nonspecific plant defence. *Curr. Opin. Plant Biol.* 3:315-319.
- Holst, O., and Brade, H. 1992. Chemical structure of the core region of lipopolysaccharides. Pages 135-170 in: *Bacterial Endotoxins Lipopolysaccharide*. D. C. Morrison and J. C. Ryan, eds. CRC Press. Boca Raton, FL, U.S.A.
- Holst, O. 2002. Chemical structure of the core region of lipopolysaccharides—An update. *Trends Glycosci. Glycotech.* 14:87-103.
- Hötte, B., Rath-Arnold, I., Pühler, A., and Simon, R. 1990. Cloning and analysis of a 35.3-kilobases DNA region involved in exopolysaccharide production by *Xanthomonas campestris* pv. *campestris*. *J. Bacteriol.* 172:2804-2807.
- Imler, J. L., and Zheng, L. 2003. Biology of toll receptors: Lessons from insects and mammals. *J. Leukocyte Biol.* 74:1-9.
- Jack, R. S., Fan, X., Bernheiden, M., Rune, G., Ehlers, M., and Weber, A. 1997. Lipopolysaccharide-binding protein is required to combat a murine gram-negative bacterial infection. *Nature* 389:742-745.
- Jann, K., Jann, B., Orskov, F., Orskov, I., and Westphal, O. 1965. Immunological studies of K antigens from *Escherichia coli*. II. K antigen from *E. coli* O8:K42(A):H-. *Biochem. Z.* 342:1-22.
- Jiang, M., and Zhang J. 2003. Cross-talk between calcium and reactive oxygen species originated from NADPH oxidase in abscisic acid-induced antioxidant defence in leaves of maize seedlings. *Plant Cell Environ.* 26:929-939.
- Kittelberger, R., and Hilbink, F. 1993. Sensitive silver-staining detection of bacterial lipopolysaccharide and extracellular polysaccharide. *Biophys. Methods* 26:81-86.
- Köplin, R., Arnold, W., Hotte, B., Simon, R., Wang, G. and Pühler A. 1992. Genetics of xanthan production in *Xanthomonas campestris*: The *xanA* and *xanB* genes are involved in UDP-glucose and GDP-mannose biosynthesis. *J. Bacteriol.* 174:191-199.
- Köplin, R., Wang, G. Hotte, B. Priefer, U. B., and Pühler, A. 1993. A 3.9-kb DNA region of *Xanthomonas campestris* pv. *campestris* that is necessary for lipopolysaccharide production encodes a set of enzymes involved in the synthesis of dTDP-rhamnose. *J. Bacteriol.* 175:7786-7792.
- Lesse, A. J., Campagnari, A. A., Bittner, W. E., and Apicella, M. A. 1990. Increased resolution of lipopolysaccharides utilizing trichine-sodium dodecyl sul fate-polyacrylamide gel electrophoresis. *J. Immunol. Methods* 126:109-117.
- McGuinness, D. H., Dehal, P. K., and Pleass, R. J. 2003. Pattern recognition molecules and innate immunity to parasites. *Trends Parasitol.* 19:312-319.
- Meyer, A., Pühler, A., and Niehaus, K. 2001. The lipopolysaccharides of the phytopathogen *Xanthomonas campestris* pv. *campestris* induce an oxidative burst reaction in cell cultures of *Nicotiana tabacum*. *Planta* 213:214-222.
- Mithöfer, A., Ebel, J., Bhagwat, A. A., Boller, T., and Neuhaus-Url, G. 1999. Transgenic aequorin monitors cytosolic calcium transients in soybean cells challenged with β-glucan or chitin elicitors. *Planta* 207:566-574.
- Molinaro, A., Silipo, A., Lanzetta, R., Newman, M. A., Dow, J. M., and Parilli, M. 2003. Structural elucidation of the O-chain of the lipopolysaccharide from *Xanthomonas campestris* strain 8004. *Carbohydr. Res.* 338:277-281.

- Morris, E. R., and Walker, J. C. 2003. Receptor-like protein kinases: The keys to response. *Curr. Opin. Plant Biol.* 6:339-342.
- Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Newman, M.-A., Daniels, M. J., and Dow, J. M. 1997. The activity of lipid A and core components of bacterial lipopolysaccharides in the prevention of the hypersensitive response in pepper. *Mol. Plant-Microbe Interact.* 10:926-928.
- Newman, M.-A., von Roepenack-Lahaye, E., Parr, A., Daniels, M. J., and Dow, J. M. 2002. Prior exposure to lipopolysaccharide potentiates expression of plant defenses in response to bacteria. *Plant J.* 29:487-495.
- Nürnberg, T., and Brunner, F. 2002. Innate immunity in plants and animals: Emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. *Curr. Opin. Plant Biol.* 5:318-324.
- Nürnberg, T., Brunner, F., Kemmerling, B., and Piater, L. 2004. Innate immunity in plants and animals: Striking similarities and obvious differences. *Immunol. Rev.* 198:249-266.
- Ojanen, T., Helander, I. M., Haahtela, K., Korhonen, T. K., and Laakso, T. 1993. Outer-membrane proteins and lipopolysaccharides in pathovars of *Xanthomonas campestris*. *App. Environ. Microbiol.* 59:4143-4151.
- Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Du Huffel, C. V. X., and Birdwell, D. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutation in *Tlr4* gene. *Science* 282:2085-2088.
- Qureshi, N., Mascagni, P., Ribi, E., and Takayama, K. 1985. Monophosphoryl lipid A obtained from lipopolysaccharides of *Salmonella-Minnesota* R595—Purification of the dimethyl derivative by high-performance liquid-chromatography and complete structural determination. *J. Biol. Chem.* 260:5271-5278.
- Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P., and Malo, D. 1999. Endotoxin-tolerant mice have mutations in toll-like receptor 4 (*Tlr4*). *J. Exp. Med.* 189:615-625.
- Radman, R., Saez, T., Bucke, C., and Keshavarz, T. 2003. Elicitation of plants and microbial cell systems. *Biotech. Appl. Biochem.* 37:91-102.
- Raetz, C. R. H., and Whitfield, C. 2002. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71:635-700.
- Scheidle, H., Groß, A., and Niehaus, K. 2005. The Lipid A substructure of the *Sinorhizobium meliloti* lipopolysaccharide is sufficient to suppress the oxidative burst in host plants. *New Phytol.* 165:559-566.
- Shiu, S.-H., and Bleecker, A. 2003. Expansion of the receptor-like kinase/pelle gene family and receptor-like proteins in Arabidopsis. *Plant Physiol.* 132:530-543.
- Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19. *Gene* 145:69-73.
- Steinmann, D. 1996. Beteiligung von exo- und lipopolysacchariden an der Interaktion von *Xanthomonas campestris* pv. *campestris* mit der Wirtspflanze *Brassica oleracea* und der nicht-Wirtspflanze *Capsicum annuum*. Ph.D. thesis. Universität Bielefeld, Bielefeld, Germany.
- Stevenson, G., Neal, B., Liu, D., Hobbs, M., Packer, N. H., Batley, M., Redmond, J. W., Lindquist, L., and Reeves, P. 1994. Structure of the O-antigen of *Escherichia coli* K-12 and the sequence of its *rfb* gene-cluster. *J. Bacteriol.* 176:4144-4156.
- Takayama, K., Qureshi, N., Hyver, K., Honovich, J., Cotter, R. J., Mascagni, P., and Schneider, H. 1986. Characterization of a structural series of lipid-A obtained from the lipopolysaccharides of *Neisseria-Gonorrhoeae*—Combined Laser desorption and fast-atom-bombardment mass-spectral analysis of high-performance liquid chromatography-purified dimethyl derivatives. *J. Biol. Chem.* 261:10624-10631.
- Trimble, R. B., and Maley, F. 1983. Glycoprotein-biosynthesis in yeast—Protein confirmation affects processing of high mannose oligosaccharide on carboxypeptidase-Y and invertase. *J. Biol. Chem.* 258:2562-2567.
- Underhill, D. M., and Ozinsky, A. 2002. Toll-like receptors: Key mediators of microbe detection. *Curr. Opin. Immunol.* 14:103-110.
- Van den Ackerveken, G., and Bonas, U. 1997. Bacterial avirulence proteins as triggers of plant disease resistance. *Trends Microbiol.* 5:394-398.
- Vorhölter, F.-J., Niehaus, K., and Pühler, A. 2001. Lipopolysaccharide biosynthesis in *Xanthomonas campestris* pv. *campestris*: A cluster of 15 genes is involved in the biosynthesis of the LPS O-antigen and the LPS core. *Mol. Genet. Genomics* 266:79-95.
- Warm, E., and Laties, G. G. 1982. Quantification of hydrogen-peroxide in plant-extracts by the chemi-luminescence reaction with luminol. *Phytochemistry* 21:827-831.
- Wurfel, M. M., Monks, B. G., Ingalls, R. R., Dedrick, R. L., Delude, R., and Zhou, D. 1997. Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact. *J. Exp. Med.* 186:2051-2056.